

Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11) **EP 1 321 527 A1**

(12) **EUROPEAN PATENT APPLICATION**  
published in accordance with Art. 158(3) EPC

(43) Date of publication:  
25.06.2003 Bulletin 2003/26

(51) Int Cl.7: **C12P 19/14, C12N 9/40,**  
**C12N 1/14**

(21) Application number: **01955615.8**

(86) International application number:  
**PCT/JP01/06848**

(22) Date of filing: **08.08.2001**

(87) International publication number:  
**WO 02/018614 (07.03.2002 Gazette 2002/10)**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU**  
**MC NL PT SE TR**

(30) Priority: **30.08.2000 JP 2000260359**

(71) Applicants:

- **Amano Enzyme Inc.**  
Nagoya-shi, Aichi 460-8630 (JP)
- **Bio Research Corporation of Yokohama**  
Yokohama-shi, Kanagawa 230-0053 (JP)
- **Ensui Sugar Refining Company, Limited**  
Yokohama-shi, Kanagawa-ken, 230-0053 (JP)

(72) Inventors:

- **OKADA, Masamichi**  
Kakamigahara-shi, Gifu 509-0108 (JP)

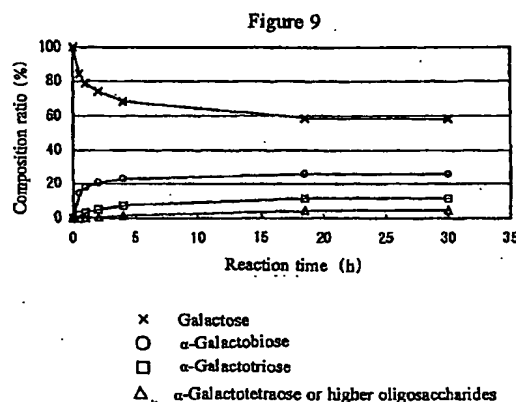
- **MORI, Shigeharu**  
Kakamigahara-shi, Gifu 509-0108 (JP)
- **HASHIMOTO, Hiroyuki**  
Shimamoto-cho, Mishima-gun, Osaka 618-00 (JP)
- **FUJITA, Koki,**  
**BIO RESEARCH CORPORATION OF**  
**YOKOHAMA**  
Yokohama-shi, Kanagawa 230-0053 (JP)
- **HARA, Kozo,**  
**BIO RESRCH CORPORATION OF YOKOHAMA**  
Yokohama-shi, Kanagawa 236-0004 (JP)

(74) Representative: **Gardner, Rebecca**  
**Frank B. Dehn & Co.**  
179 Queen Victoria Street  
London EC4V 4EL (GB)

(54) **METHOD OF ELEVATING YIELD OF OLIGOSACCHARIDES CONTAINING**  
**ALPHA-GALACTOSYL AND ANTI-CANDIDA COMPOSITIONS**

(57) A novel method of very efficiently increasing the yield of oligosaccharides containing  $\alpha$ -galactosyl, compared with the conventional methods, which comprises treating galactose or a galactose-containing material with a specific  $\alpha$ -galactosidase and thus performing a dehydrocondensation reaction at a high substrate concentration.

Anti-candida compositions originating in foods, having a high safety and excellent anti-candida effect and not being restricted in the supply, which contain as the active ingredient oligosaccharides obtained by treating galactose or a galactose-containing material with an  $\alpha$ -galactosidase and thus performing a dehydrocondensation.



## Description

### FIELD OF THE INVENTION

[0001] The present invention relates to a method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl by treating galactose or a galactose-containing material with a specific microorganism-derived galactosidase and an anti-candida composition comprising as an active ingredient an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose.

### BACKGROUND OF THE INVENTION

[0002] Recently, various saccharide-related enzymes which synthesize certain oligosaccharides were screened for on the basis of microorganisms, and the advancement in a technology for utilizing such a microorganism-derived enzyme and in a technology for purifying a resultant synthetic oligosaccharide extensively enables a large scale production of the oligosaccharide at a low cost. Accordingly, an oligosaccharide became utilizable even in a field familiar to us such as a food industry. Those known typically are a coupling sugar, fructooligosaccharide,  $\beta$ -galactooligosaccharide, soybean oligosaccharide, isomaltooligosaccharide, palatinose, lactosucrose oligosaccharide and the like.

[0003] Any of these oligosaccharides were reported to have a bifidus microorganism growth activity or a carlostatic or anti-carlogenic property as well as a mineral absorption-promoting effect, and they are utilized as a specified health-care food or a starting material thereof.

[0004] It is also reported recently that an oligosaccharide containing  $\alpha$ -galactosyl such as melibiose, manninotriose, raffinose and stachyose has a potent proliferating effect on a bifidus microorganism, has a favorable property as a food, and has a carcinostatic effect or natural killer cell-activating effect (Shigeki Motoi et al, Japanese Cancer Association (meeting report), 40, 132 (1981)), and such an oligosaccharide is considered to be extremely useful and attractive as a beverage, food product, pharmaceutical and a starting material thereof.

[0005] A known source of raffinose is a beet or soybean oligosaccharide, while that of stachyose is a soybean oligosaccharide. However, a soybean oligosaccharide is not found in abundance, and can not be supplied in a large amount. Besides, it is costly. Raffinose contained in a beet is also disadvantageous since it is available only during a period of October to March, its annual supply is only several 100 tons and its current price is so high. Melibiose and manninotriose are present in small amounts in a soybean oligosaccharide, and have the structures formed by deleting a fructose moiety from raffinose and stachyose, respectively. While they are synthesized artificially by means of the decomposition of raffinose and stachyose, they are very expensive since the starting raffinose and stachyose are also expensive.

[0006] On the other hand, an enzyme reaction employing an  $\alpha$ -galactosidase may also be employed for synthesizing an oligosaccharide containing  $\alpha$ -galactosyl. Such an enzyme reaction is classified into a saccharide transfer reaction and a dehydrocondensation reaction.

[0007] The saccharide transfer reaction is not suitable as an industrial method since it requires a synthetic substrate such as p-nitrophenyl  $\alpha$ -galactoside as an  $\alpha$ -galactosyl group donor (JP-A-10-201472) and also requires as a substrate an expensive compound containing  $\alpha$ -galactosyl such as raffinose.

[0008] When utilizing the dehydrocondensation reaction, it is possible to conduct a reaction at a high substrate concentration, and a free galactose employed as a substrate may be one obtained by a hydrolysis of a less expensive saccharide such as lactose, whereby accomplishing an economically advantageous reaction.

[0009] An enzyme which can be employed in this dehydrocondensation reaction is proposed to be an  $\alpha$ -galactosidase produced by a microorganism such as *Pycnoporus cinnabarinus*, *Streptococcus bovis*, *Diplococcus pneumoniae*, *Mortierella vinacea*, *Pseudomonas fluorescens* strain H-601 (deposition No: FERM P-11027) and *Candida guilliermondii* strain H-404 (deposition No: FERM P-11026) as well as a plant such as *Vicia sativa* and Green coffee bean (Japanese Patent No.3028258).

[0010] However, these enzymes are not applied to an industrial scale production since they exhibit low reaction rates and low reaction yields at high substrate concentrations. Thus, the  $\alpha$ -galactosidase produced by *Candida guilliermondii* strain H-404 which has been believed to exhibit the highest reaction rate and the highest reaction yield requires a period, for example, as long as about 90 hours until its reaction is plateaued when being treated with 60% (w/v) galactose under the conditions of 35U<sup>M</sup>/g-galactose and 50°C, but only gives a yield of oligosaccharides containing  $\alpha$ -galactosyl as low as about 25% (Applied Science of Saccharide, 44, 69-75, 1997).

[0011] As described above, an oligosaccharide containing  $\alpha$ -galactosyl having useful characteristics as a food material is not supplied in a large amount or at a low cost and desired to be produced on an industrial scale.

[0012] The invention is intended to solve the problem that a conventional method poses a low reaction rate and a low reaction yield in a dehydrocondensation reaction at a high substrate concentration and is less practical, and its objective is to provide a method for producing an oligosaccharide containing  $\alpha$ -galactosyl far more efficiently than any conventional method by using a certain  $\alpha$ -galactosidase.

[0013] On the other hand, a *Candida* microorganism is one of the yeasts which are persistent in the intestine of a human, and is an opportunistic pathogen inducing serious systemic infections when the resistance is re-

duced due to an infectious disease or other diseases. An intestinal *Candida* microorganism exerts its effect on an immune system via the cell itself or a *Candida* toxin produced by the microorganism, and was reported to serve as an atopic dermatitis exacerbation factor (Michio Matsuda et al., allergy clinics, 56, 768-772 (1991)) or to be involved in the allergic diseases other than the atopic dermatitis, such as bronchial asthma and rhinitis. Otherwise, it induces, when proliferating abnormally in an intestine, a health problem such as a chronic fatigue called yeast connection, headache, uneasiness, unsettledness, depression and the like.

[0014] As a method for treating such a *Candida*-induced mycosis or atopic dermatitis, an antifungal therapy is considered to be effective, and it was reported recently that a trisaccharide raffinose occurring in a beet plant has not only in vitro but also in vivo inhibitory and eradication effects on a *Candida* microorganism (JP-A-11-240837) and actually exerted an effect on an atopic dermatitis (Taizo Nagura et al, Food Industries, 2.28, 29, 1999). An oral administration of raffinose does not cause side effects observed with an antifungal agent such as a transient exacerbation of rash or digestive organ signs, and is regarded as a safer therapy against an atopic dermatitis.

[0015] Nevertheless, a further potent and further safer therapy has been demanded since the anti-candida effect of raffinose is lower than that of an antifungal agent. Also since a *Candida* microorganism has various adverse effects on a human health as described above, a food product having a more potent anti-candida effect has been desired from a viewpoint of a diet-based health control trend in response to the demand of reducing the medical expense. Another problem associated with raffinose is its limited supply and expensiveness as described above.

[0016] On the other hand, the physiological functions of an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose were also reported intensively, including a neutralizing effect of  $\alpha$ -1,3-galactobiose ( $\alpha$ -1,3 Gal2) on a toxin produced by *Clostridium difficile* which is a causative bacteria of a colitis (L.D. Heerze et al., J.Infect.Dis, 169, 1291-1296 (1994)) and an inhibitory and therapeutic effect of  $\alpha$ -1,4 galactobiose ( $\alpha$ -1,4 Gal2) on a pathogenic *E.coli* 0157 (C.A.Lingwood, Adv. Lipid Res., 25, 189-211 (1993)).

[0017] Nevertheless, no reports suggested any anti-candida effect of an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose.

[0018] Also since  $\alpha$ -galactosyl group is present only in the form of an  $\alpha$ -galactobiose ( $\alpha$ -Gal2) in a living body and the  $\alpha$ -1,6-galactosyl glucose structure possessed by raffinose does not exist while the mechanisms of the anti-candida effect of raffinose are considered to be based on the intestinal flora-improving effect, the inhibitory effect on the adhesion or settlement of a *Candida* microorganism on a digestive tract and the immune activation effect, we assumed that  $\alpha$ -Gal2 plays an impor-

tant role in the in vivo effect of raffinose and focused on an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose, whereby accomplishing the invention.

[0019] Accordingly, an objective of the invention is to provide an anti-candida composition which is derived from a food, highly safe, excellent in anti-candida effect, and can be supplied limitlessly.

## SUMMARY OF THE INVENTION

[0020] The invention relates to a method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl comprising treating galactose or a galactose-containing material with an  $\alpha$ -galactosidase derived from a specific microorganism, and such an microorganism which produces an  $\alpha$ -galactosidase employed in the invention may, for example, be a fungus such as *Aspergillus*, *Penicillium* and *Trichoderma*, yeast such as *Saccharomyces* and a bacterium such as *Bacillus*, with an  $\alpha$ -galactosidase derived from a microorganism of *Aspergillus* being preferred.

[0021] Among the microorganisms listed above, those employed preferably are *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus pulverulentus* among the fungi of *Aspergillus*, *Penicillium citrinum* and *Penicillium multicolor* among the fungi of *Penicillium* and *Trichoderma viride* among the fungi of *Trichoderma*, with *Aspergillus niger* being preferred especially.

[0022] *Saccharomyces cerevisiae* is preferred among the yeasts of *Saccharomyces*, and *Bacillus megaterium* is preferred among the bacteria of *Bacillus*.

[0023] Moreover, it was also discovered that a fungus we isolated from a soil produces a novel  $\alpha$ -galactosidase which is utilizable in the invention.

[0024] This strain has the following mycological characteristics.

Conidial top: Spherical to radial.

Conidium (optical microscope): Spherical to semi-spherical, smooth to slightly rough surface.

Conidium (electron microscope): Rough surface (protrusion), diameter (about 3.5 to 4.0  $\mu$ m).

Exudate: Slight (transparent to tan).

Odor: Almost none

Sterigmata: 2-Stage.

Colony formation rate (malt extract agar medium, incubated at 25°C): >85 mm (7-day incubation), >85 mm (12-day incubation).

Colony growth rate (Czapek's yeast agar medium, incubated at 25°C): 60 to 67 mm (7-day incubation), 65 to 73 mm (12-day incubation).

Colony color (malt extract agar medium): Black (front), colorless (back)

Colony color (Czapek's yeast agar medium): Black to grayish black (front), cream to grayish yellow (back)

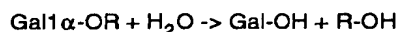
[0025] This strain is classified as *Aspergillus niger* var. *niger* based on the conidial shape, sterigmata and colony color. We designated this strain as *Aspergillus niger* strain APC-9319. This strain was deposited under

the deposition number of FERM BP-7680 to International Patent Organism Depository of National Institute of Advanced Industrial Science and Technology (IPOD) (transfer from domestic deposition to international deposition, Original deposition date: August 29, 2000, Domestic deposition number: FERM P-18003).

**[0026]** This novel  $\alpha$ -galactosidase has the following physicochemical characteristics:

**[1] Effect**

**[0027]** It catalyzes a reaction for hydrolyzing an  $\alpha$ -galactoside bond to liberate D-galactose:



wherein Gal1 $\alpha$ -OR is a saccharide containing  $\alpha$ -galactosyl, Gal-OH is a free galactose, and R-OH is a compound containing hydroxyl such as various saccharides, alcohols and phenols);

**[2] Substrate specificity**

**[0028]** It acts on a saccharide having an  $\alpha$ -galactosyl group at its non-reducing terminus such as melibiose, raffinose and stachyose as well as p-nitrophenyl  $\alpha$ -galactoside, with the relative rate at which melibiose is decomposed being about 9 based on the decomposition rate using p-nitrophenyl  $\alpha$ -galactoside as a substrate being regarded as 100;

**[3] Optimum pH and pH stability**

**[0029]** Its optimum pH is 2.5 to 6.0, and it is stable within the range from pH 3.5 to 8.0 when allowed to stand for 1 hour at 40°C;

**[4] Optimum temperature and heat stability**

**[0030]** Its optimum temperature at pH 4.5 (acetate buffer) is 60°C, and it is stable at a temperature not higher than 60°C when allowed to stand at pH 4.5 (acetate buffer) for 15 minutes;

**[5] Molecular weight and isoelectric point**

**[0031]** Its molecular weight measured by a gel filtration method using a YMC-Pack Diol-200 column (YMC) is 217,000 and its molecular weight measured by an SDS-PAGE is 117,000, while its isoelectric point measured by an isoelectric focusing is 4.2.

**[0032]** When producing an  $\alpha$ -galactosidase using any of these microorganisms, a solid culture or liquid culture is usually employed.

**[0033]** A medium for a solid culture is a wheat bran itself or a wheat bran supplemented with various additives including organic and inorganic nitrogen com-

pounds, such as KINAKO powder, soybean powder, ammonium salt, nitrate, urea, glutamic acid, aspartic acid, polypeptone, corn steep liquor, meat extract, yeast extract, protein hydrolysate and the like. In addition, suitable inorganic salts may also be added.

**[0034]** A medium for a liquid culture may for example be a synthetic medium or natural medium containing carbon sources, nitrogen sources, inorganic salts and essential nutrients which are required for a favorable growth of a relevant microorganism and a satisfactory production of an enzyme. For example, a carbon source may be a carbohydrate such as a starch or constituent thereof, roasted dextrin, processed starch, starch derivative, physically processed starch and  $\alpha$  starch or a galactose-containing material. Typically, a soluble starch, corn starch, potato starch, sweet potato starch, dextrin, amylopectin, amylose, galactose, lactose, raffinose, and the like, any of which can be employed alone or in combination with each other.

**[0035]** A nitrogen source may for example be an organic nitrogen source material such as a polypeptone, casein, meat extract, yeast extract, corn steep liquor or soybean extract or soybean lea extract, an inorganic salt nitrogen compound such as ammonium sulfate and ammonium phosphate, and an amino acid such as glutamic acid, any of which can be employed alone or in combination with each other.

**[0036]** An inorganic salt may for example be a phosphate such as monopotassium phosphate and dipotassium phosphate, a magnesium salt such as magnesium sulfate, a calcium salt such as calcium chloride and a sodium salt such as sodium carbonate, any of which can be employed alone or in combination with each other.

**[0037]** A solid culture is conducted without shaking at a pH of the culture medium within the range from 3 to 7, preferably 4 to 7, to which an inventive microorganism is inoculated and cultured at 10 to 40°C, preferably 20 to 37°C for 1 to 10 days. After incubation, the culture medium extract was subjected to an ethanol precipitation and the like to obtain  $\alpha$ -galactosidase as a crude enzyme precipitate.

**[0038]** A liquid culture is conducted with shaking or with aerating and stirring under an aerobic condition, at a pH of the culture medium within the range from 4 to 10, preferably 5 to 8 at a temperature of 10 to 40°C, preferably 25 to 37°C, for a period of 24 to 96 hours. After incubation, the cells were removed by centrifugation or other suitable solid-liquid separation means to obtain a culture supernatant. Alternatively, the cells were treated physically or enzymatically to obtain an intracellular extract.

**[0039]** Then, such a crude enzyme solution is subjected to a suitable combination of an ammonium sulfate salting out, gel filtration, hydrophobic interaction chromatography and the like to obtain a highly pure  $\alpha$ -galactosidase.

**[0040]** An enzyme used for producing an oligosaccharide containing  $\alpha$ -galactosyl may not only be the en-

zyme preparations obtained by the above-described method but also an extract of a solid culture and a culture supernatant or intracellular extract of a liquid culture which may be employed as enzyme formulations as they are. If necessary, an enzyme obtained by a known purification method may also be employed. It is also possible to use a cell itself as an enzyme formulation. Otherwise, an  $\alpha$ -galactosidase present as a contaminant in a commercially available enzyme formulation, such as a cellulase or protease formulation may also be employed, and in such a case the enzyme formulation may be used as it is or subjected to a known purification process to isolate the intended  $\alpha$ -galactosidase.

**[0041]** Examples of the commercially available enzyme formulation products employed for producing an oligosaccharide containing  $\alpha$ -galactosyl are listed below: *Aspergillus niger*-derived  $\alpha$ -Galactosidase S-DS (6,500 U/g), lipase A "AMANO" 6 (231 U/g), hemicellulase "AMANO" 90G (97 U/g) or cellulase A "AMANO" 3 (26 U/g), *Aspergillus oryzae*-derived "UMAMIZYME" (163 U/g), protease M "AMANO" (3 U/g), *Aspergillus pulverulentus*-derived pectidase G "AMANO" (131 U/g), *Penicillium citrinum*-derived protease B "AMANO" (235 U/g) or nuclease "AMANO" (388 U/g), *Trichoderma viride*-derived cellulase "AMANO" 4 (6 U/g) and the like.

**[0042]** Among those listed above, *Aspergillus niger*-derived  $\alpha$ -Galactosidase S-DS (6,500 U/g), *Penicillium citrinum*-derived protease B "AMANO" (235 U/g) and nuclease "AMANO" (388 U/g) are preferred.

**[0043]** All of the commercially available enzyme formulations described above are the products of AMANO ENZYME, and the number of each trade name represents the activity level of the  $\alpha$ -galactosidase contained. The activity was measured by the method in Reference Example 1 described below using an acetate buffer, pH6.0.

**[0044]** Such an enzyme or a cell which produces such an enzyme can be immobilized and utilized repetitively in the reaction in a continuous or batch process.

**[0045]** A starting material to be subjected to an  $\alpha$ -galactosidase reaction may for example be galactose or a galactose-containing material. Those exemplified typically are galactose, a mixture of galactose and glucose, a hydrolysate of a galactose-containing compound such as lactose and the like, any of which can be employed alone or in combination with each other. The galactose is not only a commercially available galactose but also a galactose obtained by hydrolyzing a naturally occurring or synthetic oligosaccharide containing  $\alpha$ -galactosyl or  $\beta$ -galactosyl, glucoside or polysaccharide such as melibiose, manninotriose, raffinose, stachyose, planteose, verbascose, galactans, galactomannan, arabinogalactan, rhamnogalactan, galactolipid, ferulated galactose, galactopinitol, galactosylglycerol, galactinol, lactose, lactitol, lactulose, galactooligosaccharide and the like with an enzyme ( $\beta$ -galactanase,  $\beta$ -galactosidase,  $\alpha$ -galactosidase and the like) or an acid.

**[0046]** The ratio of galactose and glucose in the mix-

ture is not limited particularly, as far as galactose is contained.

**[0047]** Glucose, which is to be combined with galactose, is not only a commercially available glucose but also a glucose obtained by hydrolyzing a naturally occurring or synthetic oligosaccharide containing  $\alpha$ -glucosyl or  $\beta$ -glucosyl, glucoside or polysaccharide such as starch, maltooligosaccharide, isomaltoligosaccharide, nigerooligosaccharide, kojic oligosaccharide, cyclodextrin, trehalose, maltitol, cellulose, cellobiosaccharide, sophorooligosaccharide, laminaribiosaccharide, gentiobiosaccharide and the like with an enzyme (amylase, glucoamylase, cellulase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase and the like) or an acid. The compound which is to be combined with galactose is not limited to glucose, and may be any other saccharide.

**[0048]** As a hydrolysate of a compound containing galactose, a material obtained by hydrolyzing a naturally occurring or synthetic oligosaccharide containing  $\alpha$ -galactosyl or  $\beta$ -galactosyl, glucoside or polysaccharide such as melibiose, manninotriose, raffinose, stachyose, planteose, verbascose, galactans, galactomannan, arabinogalactan, rhamnogalactan, galactolipid, ferulated galactose, galactopinitol, galactosylglycerol, galactinol, lactose, lactitol, lactulose, galactooligosaccharide and the like with an enzyme ( $\beta$ -galactanase,  $\beta$ -galactosidase,  $\alpha$ -galactosidase and the like) or an acid can be utilized as it is. Among those listed above, a lactose hydrolysate obtained by hydrolyzing a less expensive lactose with ( $\beta$ -galactosidase or an acid is utilized preferably as it is. In addition, a lactose hydrolysate may be supplemented with galactose or glucose described above, or its ratio between galactose and glucose can be changed using an ion exchange chromatography, an activated charcoal column and the like.

**[0049]** While an  $\alpha$ -galactosidase is a hydrolytic enzyme naturally, it catalyzes a dehydrocondensation reaction, which is a reverse reaction of the hydrolysis, at an increased level of galactose as a starting material which serves as a substrate. Accordingly, when galactose at a high level is treated with an  $\alpha$ -galactosidase, oligosaccharides containing  $\alpha$ -galactosyl which are compositions consisting of various oligosaccharides such as disaccharides, trisaccharides and tetrasaccharides having a structure represented by  $\alpha$ -(Gal)<sub>n</sub> (wherein n is usually an integer of 2 to 10, Gal is galactose) are formed. The site where an  $\alpha$ -galactosyl group is bound at the non-reducing terminus of such an oligosaccharide containing  $\alpha$ -galactosyl is exemplified below.

[1]  $\alpha$ -(Gal)<sub>n</sub> containing an  $\alpha$ -1,6-galactosyl group at the non-reducing terminus (wherein n is usually an integer of 2 to 10).

[2]  $\alpha$ -(Gal)<sub>n</sub> containing an  $\alpha$ -1,3-galactosyl group at the non-reducing terminus (wherein n is usually an integer of 2 to 10).

[3]  $\alpha$ -(Gal)<sub>n</sub> containing an  $\alpha$ -1,2-galactosyl group at the non-reducing terminus (wherein n is usually an

integer of 2 to 10).

[4]  $\alpha$ -(Gal) $n$  containing an  $\alpha$ -1,4-galactosyl group at the non-reducing terminus (wherein  $n$  is usually an integer of 2 to 10).

The number of the bonds ( $n$ ) is preferably 2 to 8, more preferably 2 to 6.

When glucose is present in the reaction system, an oligosaccharide containing  $\alpha$ -galactosyl having a structure represented by  $\alpha$ -(Gal) $n$ -Glc (wherein  $n$  is usually an integer of 1 to 9, Glc is glucose) resulting from the binding between galactose and glucose is formed in addition to an oligosaccharide having a structure represented by  $\alpha$ -(Gal) $n$ . The site where an  $\alpha$ -galactosyl group is bound at the non-reducing terminus of such an oligosaccharide containing  $\alpha$ -galactosyl is exemplified below.

[5]  $\alpha$ -(Gal) $n$ -Glc containing an  $\alpha$ -1,6-galactosyl group at the non-reducing terminus (wherein  $n$  is usually an integer of 1 to 9).

[6]  $\alpha$ -(Gal) $n$ -Glc containing an  $\alpha$ -1,3-galactosyl group at the non-reducing terminus (wherein  $n$  is usually an integer of 1 to 9).

[7]  $\alpha$ -(Gal) $n$ -Glc containing an  $\alpha$ -1,2-galactosyl group at the non-reducing terminus (wherein  $n$  is usually an integer of 1 to 9).

[8]  $\alpha$ -(Gal) $n$ -Glc containing an  $\alpha$ -1,4-galactosyl group at the non-reducing terminus (wherein  $n$  is usually an integer of 1 to 9).

[0050] The number of the bonds ( $n$ ) is preferably 1 to 8, more preferably 1 to 6.

[0051] In the procedure described above, the separation of an oligosaccharide from an enzyme reaction solution after completion of the reaction can be accomplished by a known separation means such as an ion exchange chromatography, activated charcoal column chromatography, gel filtration column chromatography and the like.

[0052] Since the advancement of an enzyme reaction is accompanied with a saccharide transfer reaction due to the  $\alpha$ -galactosidase-catalyzed re-decomposition of an oligosaccharide containing  $\alpha$ -galactosyl which was synthesized

previously by a dehydrocondensation reaction, the saccharide transfer reaction also contributes to the synthesis of the oligosaccharide containing  $\alpha$ -galactosyl.

[0053] The site and the number of the binding of galactose in a resultant compound or the ratio of such a compound may vary depending on the ratio between galactose and glucose in the starting material, origin of the enzyme employed or reaction conditions.

[0054] While the conditions under which an  $\alpha$ -galactosidase is reacted may vary depending on the enzyme employed, the reaction pH is 3.0 to 10.0, preferably 4.0 to 9.0.

[0055] The reaction temperature is preferably high in view of the solubility and the reaction rate, and usually 20 to 90°C, preferably 40 to 80°C. The reaction time

may vary depending on the amount of an enzyme employed, and is usually 1 to 150 hours. Nevertheless, the invention is not limited to the conditions or reaction modes described above.

[0056] When an oligosaccharide containing  $\alpha$ -galactosyl of the invention is produced, a higher concentration of galactose as a starting material is more preferred, and galactose or glucose may precipitate in the reaction system, or be present in a supersaturated condition, and used as a concentration usually of 5 to 110% (w/v), preferably 40 to 110% (w/v).

[0057] For the purpose of an efficient production of an oligosaccharide containing  $\alpha$ -galactosyl by increasing galactose concentration substantially, galactose is treated with an  $\alpha$ -galactosidase to effect a dehydrocondensation reaction as a preliminary reaction and thereafter the reaction solution is concentrated under reduced pressure prior to the dehydrocondensation reaction as a main reaction. This reaction here may be conducted in a system separate from the preliminary reaction by isolating the concentrated solution, or may be conducted in the preliminary reaction system without isolating the concentrated solution.

[0058] When the level of galactose is increased at the stage of the initial charging, galactose undergoes precipitation, which may lead to a reduction in the yield of an oligosaccharide containing  $\alpha$ -galactosyl. Nevertheless, the inventive method in which the galactose level is increased substantially to effect a dehydrocondensation reaction as described above prevents any precipitation of galactose or any inactivation of the enzyme due to the foaming upon concentration and promotes the dehydrocondensation reaction markedly, thus allowing an oligosaccharide containing  $\alpha$ -galactosyl to be produced efficiently within a short period of time at a high yield. An enzyme employed in this production method is preferably one having a higher catalytic activity of the dehydrocondensation reaction, such as those derived from various microorganisms employed in an inventive yield increasing method among which the  $\alpha$ -galactosidase obtained from *Aspergillus niger* strain APC-9319 (FERM BP-7680) is particularly preferred and can produce an oligosaccharide containing  $\alpha$ -galactosyl efficiently. The concentration under reduced pressure can be accomplished by any known means for concentration under reduced pressure such as a rotary evaporator and a vacuum crystallization container.

[0059] The invention also relates to an anti-Candida composition comprising as an active ingredient an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose which is synthesized by treating galactose or a galactose-containing material with an  $\alpha$ -galactosidase followed by a dehydrocondensation reaction.

[0060] While the origin or type of a microorganism which produces an  $\alpha$ -galactosidase employed for synthesizing an oligosaccharide containing  $\alpha$ -galactosyl of the invention is not limited particularly, and may be one derived from a plant such as a green coffee, any of var-

ious  $\alpha$ -galactosidases derived from the microorganisms employed in an inventive yield increasing method, especially an  $\alpha$ -galactosidase derived from *Aspergillus niger* strain APC-9319 (FERM BP-7680), serves to increase the yield of the oligosaccharide containing  $\alpha$ -galactosyl, whereby enabling a stable and less expensive supply of a food-derived highly safe anti-Candida composition. In addition, the synthesis may be accomplished also by treating the galactose described above with an  $\alpha$ -galactosidase to effect a dehydrocondensation reaction as a preliminary reaction followed by concentrating the reaction solution under reduced pressure prior to the dehydrocondensation reaction as a main reaction.

**[0061]** An oligosaccharide containing  $\alpha$ -galactosyl of the invention may be an oligosaccharide represented by  $\alpha$ -(Gal) $_n$  (wherein  $n$  is usually an integer of 2 to 10, Gal is galactose) consisting only of galactose or an oligosaccharide represented by  $\alpha$ -(Gal) $_n$ -Glc (wherein  $n$  is usually an integer of 1 to 9, Glc is glucose) consisting of other saccharide except for raffinose, such as galactose and glucose.

**[0062]** An anti-Candida composition of the invention can be employed as a solid, semi-solid or liquid formed by combining an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose with a customary inorganic or organic carrier or excipient. While an ordinary administration mode is an oral administration, a parenteral dosage form such as a dermal formulation can also be employed. An oral dosage form may be various formulations such as powder, granule, tablet, capsule, syrup and the like, and the formulation may contain pharmaceutically acceptable known additives such as an excipient, stability, binder, lubricant, disintegrant, suspending agent, solubilizer, flavor, sweetener and the like.

**[0063]** The dose of an anti-Candida composition of the invention is usually 1 to 15 g per day, preferably 3 to 15 g per day as an oligosaccharide containing  $\alpha$ -galactosyl as an active ingredient when given orally to an adult. Since the oligosaccharide containing  $\alpha$ -galactosyl as an active ingredient is produced from a material consumed as a food by a method applied to a food product and has no problem toxicologically and exhibited no acute toxicity in rats when given orally at 5000 mg per kg of body weight, its dose may be increased or decreased as appropriate depending on the condition, age, body weight, dosage form, administration mode and the like.

**[0064]** An anti-Candida composition of the invention can be applied to a mammal other than human such as cattle and horse as well as a laboratory animal such as rat and mouse, to which an oligosaccharide containing  $\alpha$ -galactosyl whose purity is lower may also be given.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0065]** Figure 1 shows the action pH and the optimum pH of the  $\alpha$ -galactosidase obtained in EXAMPLE 1. Fig-

ure 2 shows the pH stability of the  $\alpha$ -galactosidase obtained in EXAMPLE 1. Figure 3 shows the active temperature and the optimum temperature of the  $\alpha$ -galactosidase obtained in EXAMPLE 1. Figure 4 shows the thermostability of the  $\alpha$ -galactosidase obtained in EXAMPLE 1. Figure 5 shows the results of the measurement of the molecular weight of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 measured by an HPLC. Figure 6 shows the calibration curve in the measurement of the molecular weight of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 by an HPLC.

**[0066]** Figure 7 shows the results of the measurement of the molecular weight of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 measured by a SDS-PAGE. Figure 8 shows the isoelectric point, according to the isoelectric focusing, of the  $\alpha$ -galactosidase obtained in EXAMPLE 1. Figure 9 shows the change with time in the dehydrocondensation reaction starting from galactose in EXAMPLE 2. Figure 10 shows the composition of the oligosaccharide containing  $\alpha$ -galactosyl obtained in EXAMPLE 2. Figure 11 shows the composition of the oligosaccharide containing  $\alpha$ -galactosyl obtained in EXAMPLE 4. Figure 12 shows the change with time in the dehydrocondensation reaction starting from galactose in EXAMPLE 5. Figure 13 shows the change in the oligosaccharide employing as a starting material the galactose in EXAMPLE 6. Figure 14 shows the viable cell counts of a *Candida* microorganism in cecum and various colonic positions. "\*" indicates a significant difference observed at a significance level less than 5%.

#### BEST MODE FOR CARRYING OUT THE INVENTION

**REFERENCE EXAMPLE 1** (Measurement of activity of  $\alpha$ -galactosidase using p-nitrophenyl  $\alpha$ -galactoside as substrate)

**[0067]** 0.2 ml of 10 mM p-nitrophenyl  $\alpha$ -galactoside and 0.2 ml of a predetermined 40 mM buffer (pH in accordance with the optimum pH of the enzyme) were combined with 0.05 ml of an  $\alpha$ -galactosidase solution and reacted at 40°C for 10 minutes. After reaction, 0.5 ml of 0.2 M sodium carbonate was added to terminate the reaction, and the liberated p-nitrophenol was assayed by measuring the absorbance at 400 nm using a spectrophotometer.

**[0068]** One enzyme activity unit (U) was defined as the level of the enzyme capable of liberating 1 micromole of p-nitrophenol within 1 minute under the condition described above.

**REFERENCE EXAMPLE 2** (Measurement of activity of  $\alpha$ -galactosidase using melibiose as substrate)

**[0069]** 0.2 ml of 10 mM melibiose and 0.2 ml of a predetermined 40 mM buffer (pH in accordance with the optimum pH of the enzyme) were combined with 0.05 ml of an  $\alpha$ -galactosidase solution and reacted at 40°C

for 10 minutes. Then the mixture was heated at 100°C for 10 minutes to terminate the reaction, and the resultant glucose level was quantified using an F-kit manufactured by ROCHE-DIAGNOSTICS (glucose/fructose) or a high pressure liquid chromatography (HPLC).

[0070] One enzyme activity unit (U<sup>M</sup>) was defined as the level of the enzyme capable of producing 1 micromole of glucose within 1 minute under the condition described above.

#### EXAMPLE 1 (preparation of α-galactosidase)

[0071] 90 ml of a liquid medium (pH6.0) containing 5% wheat bran was placed in a 500 ml Sakaguchi flask, sterilized by a standard method, inoculated with *Aspergillus niger* strain APC-9319 (FERM BP-7680) and pre-incubated (seed incubation) at 25°C for 3 days. 500 g of the bran was combined with 400 ml of water, sterilized, inoculated with 10 ml of the pre-incubated culture, stirred thoroughly, and subjected to a main incubation at 25°C for 4 days.

[0072] After incubation, the bran malt (koji) was pulverized, combined with 8 L of water to extract overnight at 4°C, filtered through a filter paper to obtain an extract filtrate. The resultant extract filtrate was examined for the α-galactosidase activity, which was revealed to be 3U in 1 ml of the extract filtrate. 6 L of the extract filtrate was concentrated to 1 L using an ultrafiltration membrane (SIP manufactured by ASAHI KASEI), and combined with ammonium sulfate at a concentration giving a 70% saturation, whereby effecting a salting out.

[0073] Subsequently, the precipitate was collected by a centrifugation, dissolved in 500 ml of water, concentrated to 100 ml using an ultrafiltration membrane, combined further with 500 ml of water, concentrated to 100 ml, this procedure being repeated 3 times, whereby effecting a desalting. After desalting followed by a lyophilization, a freeze-dried powder (250 U/g) was obtained.

[0074] The extract filtrate obtained by the above-described method was combined with ammonium sulfate at a concentration giving a 70% saturation, stirred, and allowed to stand overnight at 4°C. The precipitate was collected by a centrifugation, dissolved in 10 mM phosphate buffer (pH6.0), concentrated using an ultrafiltration membrane (SIP manufactured by ASAHI KASEI), combined again with the same buffer solution, and then concentrated. This procedure was repeated three times, whereby effecting a desalting.

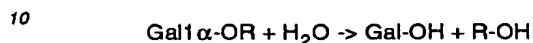
[0075] Then, an ion exchange column chromatography was conducted using a DEAE-TOYOPEARL 650 M (TOSO) equilibrated with the same buffer solution. Subsequently, a hydrophobic interaction chromatography was conducted using a butyl-TOYOPERAL 650 M (TOSO) column in 50%-saturated ammonium sulfate.

[0076] The active fractions were collected, and subjected to a gel filtration column chromatography using a TOYOPERAL HW-55S (TOSO) column equilibrated with 50mM acetate buffer (pH5.5) containing 0.3M so-

dium chloride.

[0077] As a result of these column chromatographies, an α-galactosidase whose proteins are homogeneous was obtained.

5 [0078] The α-galactosidase obtained as described above is an enzyme which catalyzes the reaction shown below by which a α-galactoside bond is hydrolyzed to liberate galactose.



wherein Gal1α-OR is a saccharide containing α-galactosyl, Gal-OH is a free galactose, and R-OH is a compound containing hydroxyl such as various saccharides, alcohols and phenols.

[0079] Then, this enzyme was employed to measure the optimum pH and the pH stability.

[0080] The enzyme activity in Figure 1 is measured at 40°C for 10 minutes using various buffer solutions (pH2.0 to 3.5: glycine-HCl buffer, pH3.5 to 6.0: acetate buffer, pH6.0 to 7.5: phosphate buffer). The enzyme activity in Figure 2 is measured as a residual activity after allowing the samples to stand at 40°C for 1 hour using various buffer solutions (pH2.5 to 3.5: glycine-HCl buffer, pH3.5 to 6.0: acetate buffer, pH6.0 to 8.0: phosphate buffer, pH8.0 to 9.0: Tris-HCl buffer). As a result, the optimum pH of this enzyme was 2.5 to 6.0, and the stability was observed within the range from 3.5 to 8.0 when allowed to stand at 40°C for 1 hour.

[0081] The optimum temperature and the thermostability of this enzyme were also measured.

[0082] In Figure 3, the enzyme activity was measured at each temperature using an acetate buffer (pH4.5). In Figure 4, the residual activity after treating at each temperature for 15 minutes was measured using the acetate buffer (pH4.5). As a result, the optimum temperature was 60°C, and the stability was observed at 60°C or below when allowed to stand for 15 minutes.

[0083] The molecular weight of this enzyme was determined by an HPLC. Using an HPLC column YMC-Pack Diol-200 produced by YMC equilibrated with a 0.1M phosphate buffer solution (pH7.0) containing 0.2M sodium chloride together with an MW-Marker (HPLC) produced by Oriental Yeast as a standard protein, the molecular weight was determined. In Figure 5, the solid line represents the absorption of the protein at 280nm, while • represents the activity of the α-galactosidase. Figure 6 shows a calibration curve, the standard proteins employed for which are glutamate dehydrogenase (250,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000) and cytochrome C (12,000) in the order of the larger molecular weight.

55 [0084] The figure in each bracket represents the respective molecular weight. As a result, the molecular weight of this enzyme was 217,000.

[0085] The molecular weight of this enzyme was de-



terminated also by an SDS-PAGE. The electrophoresis instrument employed was Phast System produced by Amersham Pharmacia Biotech. The gel employed was PhastGel Homogeneous 12.5. The molecular weight markers employed were of a HMW marker kit (Amersham Pharmacia Biotech). The standard proteins in the kit shown in Figure 7 were myosin (220,000),  $\alpha$ -2-macroglobulin (170,000),  $\beta$ -galactosidase (116,000), transferrin (76,000) and glutamate dehydrogenase (53,000) in the order of the larger molecular weight. The figure in each bracket represents the respective molecular weight. As a result, the molecular weight of this enzyme was 117,000.

**[0086]** The isoelectric point of this enzyme was determined by an isoelectric focusing. The isoelectric point was determined by running for 2 days at 500V and 4°C using \*AMPHORITE\* produced by Amersham Pharmacia Biotech (pH3.5 to 5.0). In Figure 8, the solid line represents the absorption of the protein at 280nm, while • represents the activity of the  $\alpha$ -galactosidase and ° represents the pH. As a result, the isoelectric point of this enzyme was 4.2.

**[0087]** Among the characteristics described above, the molecular weight of the  $\alpha$ -galactosidase produced by this microorganism determined by the gel filtration method (217,000) and that by the SDS-PAGE (117,000) were larger distinguishably than those of *Aspergillus niger*-produced  $\alpha$ -galactosidase reported previously, i.e., 72,000 and 69,000 (both by SDS-PAGE, Enzyme and microbial Technology, 22:383-390, 1998).

#### EXAMPLE 2 (Production1 of oligosaccharide containing $\alpha$ -galactosyl)

**[0088]** 100 ml of a buffer solution, pH4.5, containing 60 g of a galactose (WAKO PURE CHEMICAL) and 2,100 U<sub>M</sub> of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 (galactose concentration: 60% (w/v), enzyme concentration 35U<sub>M</sub>-galactose) was prepared, and allowed to react at 50°C for 30 hours. The change with time during the reaction is shown in Figure 9.

**[0089]** The reaction solution was loaded onto an activated charcoal column, from which the galactose was eluted with water and the oligosaccharide was eluted with the gradient of ethyl alcohol from 0 to 30%. The eluted oligosaccharide fraction was concentrated into dryness to obtain 24 g of an oligosaccharide containing  $\alpha$ -galactosyl. This oligosaccharide produced only galactose when hydrolyzed with an  $\alpha$ -galactosidase or an acid.

**[0090]** This oligosaccharide containing  $\alpha$ -galactosyl was subjected to an HPLC analysis (Figure 10) using a Shim-pack SCR-101 column (SHIMADZU) and revealed to consist of 11% of oligosaccharides of  $\alpha$ -galactotetraose or higher saccharides, 27% of  $\alpha$ -galactotriose and 62% of  $\alpha$ -galactobiose. The reaction product was labeled with an aminobenzoic acid ethyl ester and analyzed on an ODS column, which revealed the bind-

ing position isomer composition of the  $\alpha$ -galactobiose of  $\alpha$ -1,6 bond:  $\alpha$ -1,3-bond:  $\alpha$ -1,2bond:  $\alpha$ -1,4 bond = 44:10:5.5:1.0.

#### 5 COMPARATIVE 1 (production2 of oligosaccharide containing $\alpha$ -galactosyl)

**[0091]** Using an  $\alpha$ -galactosidase produced by *Candida guilliermondii* strain H-404 which is known to have the highest dehydrocondensation reaction-catalyzing activity among the  $\alpha$ -galactosidases instead of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 and the condition similar to that in EXAMPLE 2, an oligosaccharide was produced.

**[0092]** As a result, it took 90 hours to observe the reaction plateau (vs 30 hours in EXAMPLE 2) in spite of the enzymatic action on melibiose as a substrate similar to that of the  $\alpha$ -galactosidase obtained in Example 1, and the yield of the oligosaccharide containing  $\alpha$ -galactosyl was 14 g (vs 24 g in EXAMPLE 2).

**[0093]** Based on the results described above, the  $\alpha$ -galactosidase of the invention obtained in EXAMPLE 1 was proven to have an extremely high enzymatic activity of the dehydrocondensation reaction using a galactose as a substrate, and to be able to produce the oligosaccharide containing  $\alpha$ -galactosyl at a high yield within a short period of time.

#### 30 EXAMPLE 3 (Production3 of oligosaccharide containing $\alpha$ -galactosyl)

**[0094]** 15 L of a sample containing 9.0 kg of a galactose (WAKO PURE CHEMICAL) and 315,000 U<sub>M</sub>/g of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 (galactose concentration: 60% (w/v), enzyme concentration 35U<sub>M</sub>-galactose, pH adjusted at 4.5 with HCl) was prepared, and subjected to a preliminary reaction for 3 hours at 60°C in a vacuum crystallization container (Crystallization instrument, Model DP, manufactured by TSUKISHIMA KIKAI), and then the reaction solution was concentrated under reduced pressure at 60°C to 10 L (galactose concentration: 90% (w/v)) and then the concentrate was transferred into a trough crystallization promoter (TSUKISHIMA KIKAI), to which an  $\alpha$ -galactosidase whose enzyme concentration was 35U<sub>M</sub>/g-galactose was added and the main reaction was effected at 60°C for 30 hours. The reaction solution was loaded onto an ion exchange column (ligand exchange column having calcium as a ligand), from which the oligosaccharide and the galactose were eluted with water in this order. The eluted oligosaccharide fraction was concentrated into dryness to obtain 5.1 kg of the oligosaccharide containing  $\alpha$ -galactosyl.

**[0095]** By this production method, the substrate can be used at an extremely high concentration (final concentration: 90%), which promotes the dehydrocondensation reaction of the  $\alpha$ -galactosidase extremely, thus enabling an efficient production of an oligosaccharide

containing  $\alpha$ -galactosyl at a high yield within a short period of time, which is suitable especially for an industrial scale production.

[0096] The resultant oligosaccharide containing  $\alpha$ -galactosyl produced only galactose when hydrolyzed with an  $\alpha$ -galactosidase. This oligosaccharide containing  $\alpha$ -galactosyl was subjected to an HPLC analysis and revealed to consist of 13% of oligosaccharides of  $\alpha$ -galactotetraose or higher saccharides, 31% of  $\alpha$ -galactotriose and 56% of  $\alpha$ -galactobiose. The binding position of the  $\alpha$ -galactosyl group at the reducing terminus was mainly of  $\alpha$ -1,6 bond.

EXAMPLE 4 (Production 4 of oligosaccharide containing  $\alpha$ -galactosyl)

[0097] 100 g of a lactose (WAKO PURE CHEMICAL) was hydrolyzed with a commercially available  $\beta$ -galactosidase (LACTOZYME produced by NOVONORDISC BIOINDUSTRIES) to obtain a mixture of equal amounts of a galactose and a glucose. 100 ml of an acetate buffer, pH4.5, containing 85 g of the equal amount mixture of the galactose and the glucose obtained above and 1,500 U<sup>M</sup> of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 (saccharide concentration: 85% (w/v), enzyme concentration 35U<sup>M</sup>/g-galactose) was prepared, and allowed to react at 50°C for 87 hours.

[0098] From the reaction solution, 27 g of an oligosaccharide containing  $\alpha$ -galactosyl was obtained by a column chromatography on an activated charcoal. This oligosaccharide containing  $\alpha$ -galactosyl produced only the galactose and the glucose, when hydrolyzed with an acid or  $\alpha$ -galactosidase, with the ratio between the galactose and the glucose being about 7:3. This oligosaccharide containing  $\alpha$ -galactosyl subjected to an HPLC analysis (Figure 11) and revealed to consist of 8.0% of trisaccharides or higher oligosaccharides, 48% of  $\alpha$ -galactosyl glucose and 44% of  $\alpha$ -galactobiose.

COMPARATIVE 2 (production 5 of oligosaccharide containing  $\alpha$ -galactosyl)

[0099] Using an  $\alpha$ -galactosidase produced by *Candida guilliermondii* strain H-404 instead of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 and the condition similar to that in EXAMPLE 4, an oligosaccharide was produced. As a result, the yield of the oligosaccharide containing  $\alpha$ -galactosyl was 16 g (vs 27 g in EXAMPLE 4).

[0100] Based on the results described above, the  $\alpha$ -galactosidase of the invention obtained in EXAMPLE 1 was proven to have an extremely high enzymatic activity of the dehydrocondensation reaction even when using a mixture of the galactose and the glucose as a substrate, and to be able to produce the oligosaccharide containing  $\alpha$ -galactosyl at a high yield.

EXAMPLE 5 (Production 6 of oligosaccharide containing  $\alpha$ -galactosyl)

[0101] 100 ml of an acetate buffer solution, pH4.5, containing 60 g of a galactose (WAKO PURE CHEMICAL) and 2,100 U<sup>M</sup> of an  $\alpha$ -galactosidase S-DS derived from *Aspergillus niger* (AMANO ENZYME) (galactose concentration: 60% (w/v), enzyme concentration 35U<sup>M</sup>/g-galactose) was prepared, and allowed to react at 50°C for 30 hours. The change during the reaction is shown in Figure 12.

[0102] The reaction solution was loaded onto an activated charcoal column, from which the galactose was eluted with water and the oligosaccharide was eluted with the gradient of ethyl alcohol from 0 to 30%. The eluted oligosaccharide fraction was concentrated into dryness to obtain 21 g of an oligosaccharide containing  $\alpha$ -galactosyl. This oligosaccharide produced only galactose when hydrolyzed with an  $\alpha$ -galactosidase or an acid. This oligosaccharide containing  $\alpha$ -galactosyl was subjected to an HPLC analysis and revealed to consist of 9.0% of oligosaccharides of  $\alpha$ -galactotetraose or higher saccharides, 25% of  $\alpha$ -galactotriose and 66% of  $\alpha$ -galactobiose.

EXAMPLE 6 (Production 7,8,9 of oligosaccharide containing  $\alpha$ -galactosyl)

[0103] 100 ml of an acetate buffer, pH4.5, containing 20 g of a galactose (WAKO PURE CHEMICAL) and each 400 U<sup>M</sup> of the  $\alpha$ -galactosidase obtained in EXAMPLE 1 or the  $\alpha$ -galactosidase purified by a known method from *Penicillium citrinum*-derived protease B "AMANO" or nuclease "AMANO" (galactose concentration: 20% (w/v), enzyme concentration 20 U<sup>M</sup>/g-galactose) was prepared, and allowed to react at 40°C. The change with time during the reaction is shown in Figure 13.

[0104] While the results indicated no difference in the trisaccharide ratio between enzymes, the disaccharide ratios after reaction for 100 hours given by the  $\alpha$ -galactosidase obtained in EXAMPLE 1, nuclease "Amano" and protease B "AMANO" were 10.0%, 11.3% and 11.1%, respectively, showing the difference. As evident from these results, there was a difference in the compositions of the galactoses having different binding numbers among the various origins of the  $\alpha$ -galactosidase even when the reaction was conducted under the identical condition, and thus the  $\alpha$ -galactosidase derived from *Penicillium citrinum* is preferable for the purpose of obtaining an oligosaccharide whose ratio of the disaccharides is high.

EXAMPLE 7 (anti-candida effect of oligosaccharide containing  $\alpha$ -galactosyl)

[0105] A *Candida* microorganism was given forcibly via an oral administration to a mouse on diet with the

oligosaccharide containing  $\alpha$ -galactosyl obtained in EXAMPLE 3 to test the persistence of the Candida microorganism in an intestinal tract, whereby assessing the anti-candida effect of the oligosaccharide containing  $\alpha$ -galactosyl.

(Methods) A test mouse was a 4-week old male mouse of Crj:CD-1 (ICR) line (NIPPON CHARLES RIVER). A preliminary housing was provided to the animal for 1 week while being allowed to access a basal diet and a distilled water ad libitum. The basal diet was a solid chow for mice (MF:

ORIENTAL YEAST). After the preliminary housing, the basal diet and the distilled water were given ad libitum continuously, and a control group fed with a sucrose, a positive control group fed with a raffinose, and a treatment group fed with an oligosaccharide containing  $\alpha$ -galactosyl were provided (5 animals per group) to conduct a forcible oral administration at 100 mg/animal via an oral gavage for two weeks on a once-a-day basis. One week after initiation of the treatment, the animals in each group received 0.2 ml of a suspension of a Candida microorganism (Candida albicans IFM40009) prepared at the density of  $5.0 \times 10^9$  cells/ml, 1 week after which the cecum and the colon of each mouse were isolated and examined for the viable Candida cell count of each organ.

[0106] The viable cell of the Candida microorganism was counted by applying a Candida microorganism suspension diluted appropriately with a physiological saline onto a Candida GS (EIKEN KAGAKU) plate medium and incubating at 37°C for 42 to 48 hours followed by counting the colonies formed. The statistical processing was conducted using a Wilcoxon's rank sum test.

(Results) The viable cell counts of the cecum and the each part of the colon 1 week after the forcible administration of the Candida microorganism suspension are shown in Figure 14. While the viable cell count of the cecum 1 week after the forcible administration of the Candida microorganism suspension was not changed in the control group or the positive control group but was lower in the treatment group, that of the colon was lower in both of the positive control group and the treatment group, with a significantly lower count being observed in the treatment group especially when compared with the control group. In the figure,  $\alpha$ -GOS is an oligosaccharide containing  $\alpha$ -galactosyl.

[0107] Based on the results, the oligosaccharide containing  $\alpha$ -galactosyl in the treatment group exhibited a potent inhibitory effect on the settlement of the Candida microorganism in the intestinal tract, and revealed to have a more excellent anti-candida effect than raffinose.

#### INDUSTRIAL APPLICABILITY

[0108] Since a method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to the present invention is capable of producing any of various oligosaccharides efficiently at a high yield within a

short period of time and is also capable of production on an industrial scale, it enables a stable and less expensive supply of oligosaccharide.

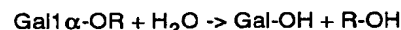
[0109] Also since an anti-candida composition of the invention has an excellent anti-candida effect and can be supplied stably, it can provide a less expensive pharmaceutical or functional food material which enables a safe amelioration or prevention of a Candida microorganism-induced mycosis or allergic disease such as an atopic dermatitis.

#### Claims

1. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl comprising treating galactose or a galactose-containing material with an  $\alpha$ -galactosidase derived from a microorganism of *Aspergillus*, *Penicillium*, *Trichoderma*, *Saccharomyces* or *Bacillus*.
2. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to Claim 1 wherein the  $\alpha$ -galactosidase is derived from a microorganism of *Aspergillus*.
3. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to Claim 2 wherein the  $\alpha$ -galactosidase is derived from *Aspergillus niger*.
4. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to Claim 3 wherein the  $\alpha$ -galactosidase has the following physicochemical characteristics:

##### [1] Effect

It catalyzes a reaction for hydrolyzing an  $\alpha$ -galactoside bond to liberate D-galactose:



wherein Gal1 $\alpha$ -OR is a saccharide containing  $\alpha$ -galactosyl, Gal-OH is a free galactose, and R-OH is a compound containing hydroxyl such as various saccharides, alcohols and phenols;

##### [2] Substrate specificity

It acts on a saccharide having an  $\alpha$ -galactosyl group at its non-reducing terminus such as melibiose, raffinose and stachyose as well as p-nitrophenyl  $\alpha$ -galactoside, with the relative rate at which melibiose is decomposed being about 9 based on the decomposition rate using p-nitrophenyl  $\alpha$ -galactoside as a substrate being regarded as 100;

##### [3] Optimum pH and pH stability

Its optimum pH is 2.5 to 6.0, and it is sta-

ble within the range from pH 3.5 to 8.0 when allowed to stand for 1 hour at 40°C;

[4] Optimum temperature and heat stability

Its optimum temperature at pH 4.5 (acetate buffer) is 60°C, and it is stable at a temperature not higher than 60°C when allowed to stand at pH 4.5 (acetate buffer) for 15 minutes;

[5] Molecular weight and isoelectric point

Its molecular weight measured by a gel filtration method using a YMC-Pack Diol-200 column (YMC) is 217,000 and its molecular weight measured by an SDS-PAGE is 117,000, while its isoelectric point measured by an isoelectric focusing is 4.2.

5. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to Claim 4 wherein the  $\alpha$ -galactosidase having the physicochemical characteristics described above is derived from *Aspergillus niger* strain APC-9319 (FERM BP-7680). 15
6. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl comprising treating the galactose or a galactose-containing material with an  $\alpha$ -galactosidase to effect a dehydrocondensation reaction as a preliminary reaction followed by concentrating the reaction solution under reduced pressure prior to the dehydrocondensation reaction as a main reaction. 25
7. A method of increasing the yield of an oligosaccharide containing  $\alpha$ -galactosyl according to Claim 6 comprising treating with an  $\alpha$ -galactosidase derived from *Aspergillus niger* strain APC-9319 (FERM BP-7680) having the physicochemical characteristics described above. 30
8. An  $\alpha$ -galactosidase produced by *Aspergillus niger* strain APC-9319 (FERM BP-7680) having the physicochemical characteristics described above. 35
9. A method of producing an  $\alpha$ -galactosidase according to Claim 8 comprising incubating *Aspergillus niger* strain APC-9319 (FERM BP-7680) in a culture medium and isolating the  $\alpha$ -galactosidase from the culture. 40
10. *Aspergillus niger* strain APC-9319 (FERM BP-7680) producing an  $\alpha$ -galactosidase according to Claim 8. 45
11. An anti-candida composition comprising as an active ingredient an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose which is synthesized by treating galactose or a galactose-containing material with an  $\alpha$ -galactosidase to effect a dehydrocondensation reaction. 50
12. An anti-candida composition comprising as an active ingredient an oligosaccharide containing  $\alpha$ -galactosyl other than raffinose which is synthesized by a method of increasing the yield according to any of Claims 1 to 7. 55
13. An anti-candida composition according to Claim 11 or 12 wherein the oligosaccharide containing  $\alpha$ -galactosyl is  $\alpha$ -(Gal)<sub>n</sub> wherein n is usually an integer of 2 to 10, and Gal represents galactose).

Figure 1

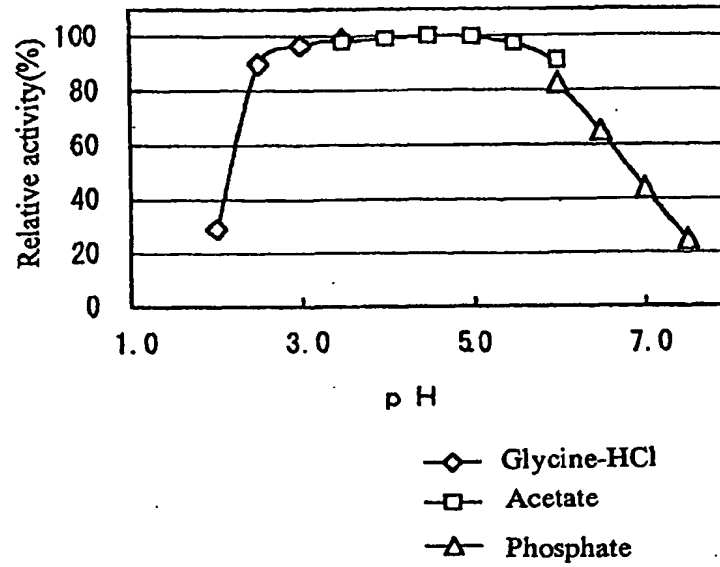


Figure 2

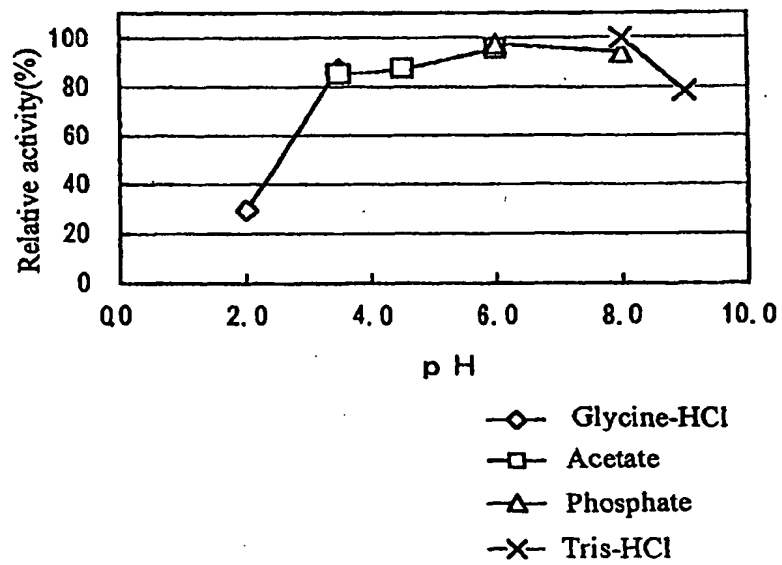


Figure 3

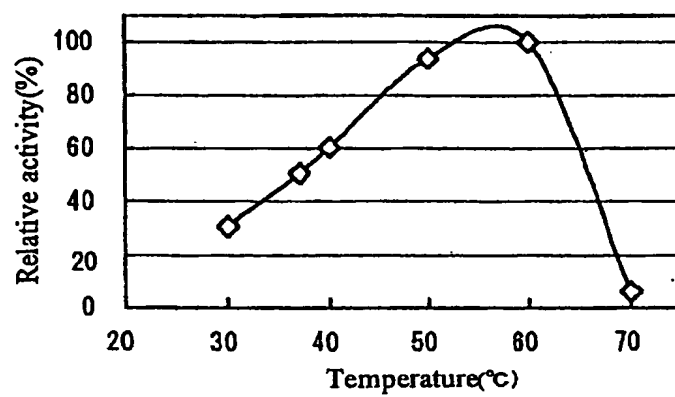


Figure 4

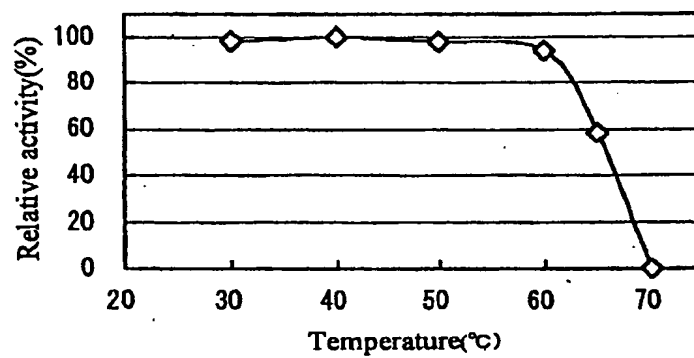


Figure 5

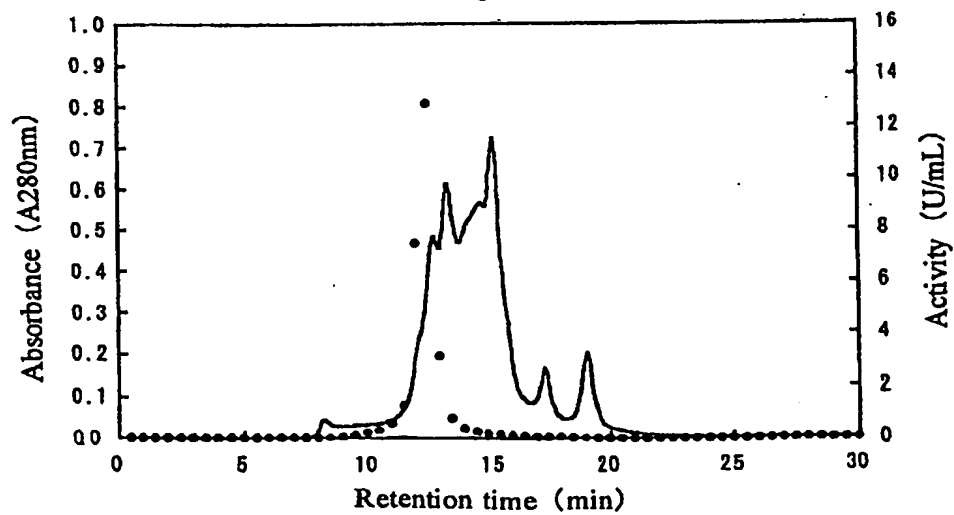


Figure 6

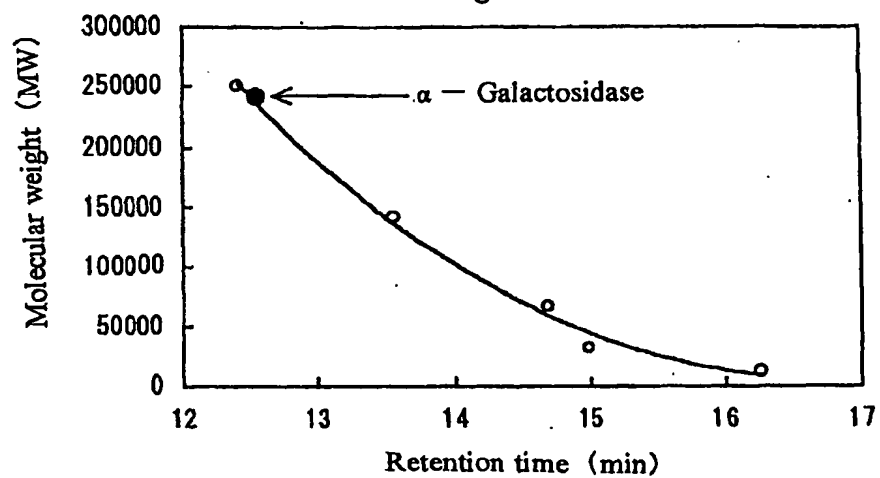


Figure 7

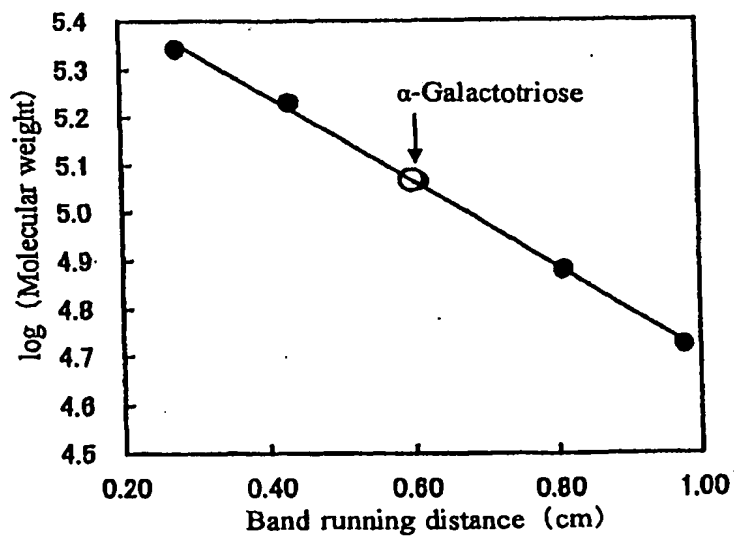


Figure 8

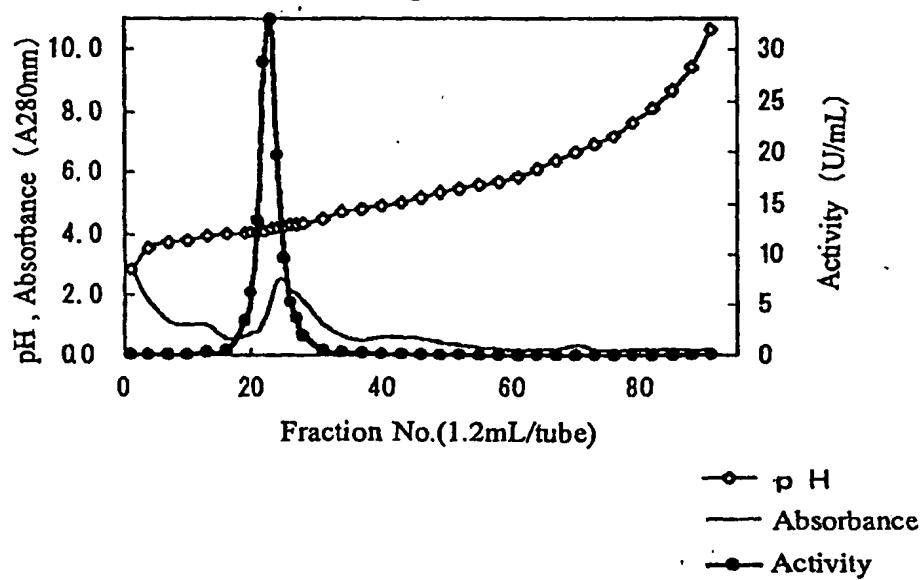


Figure 9

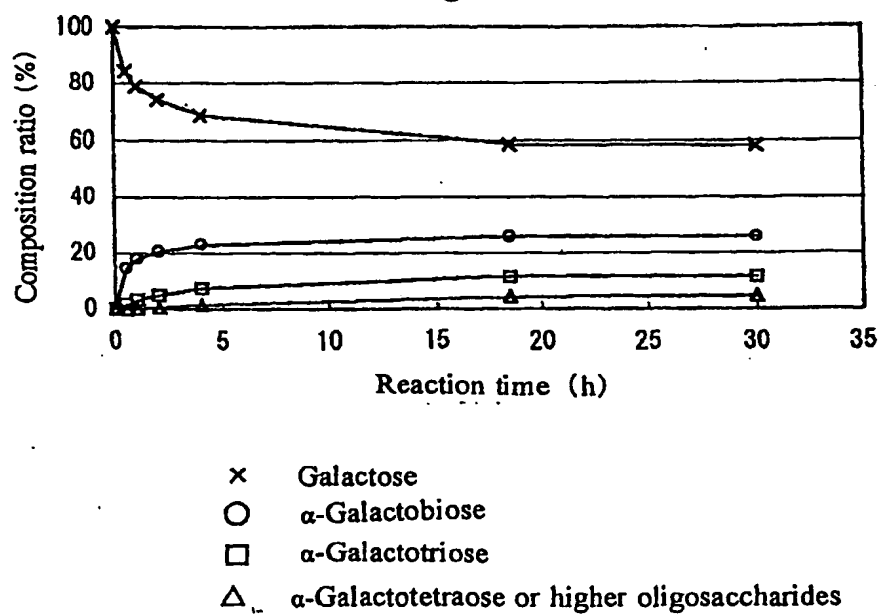
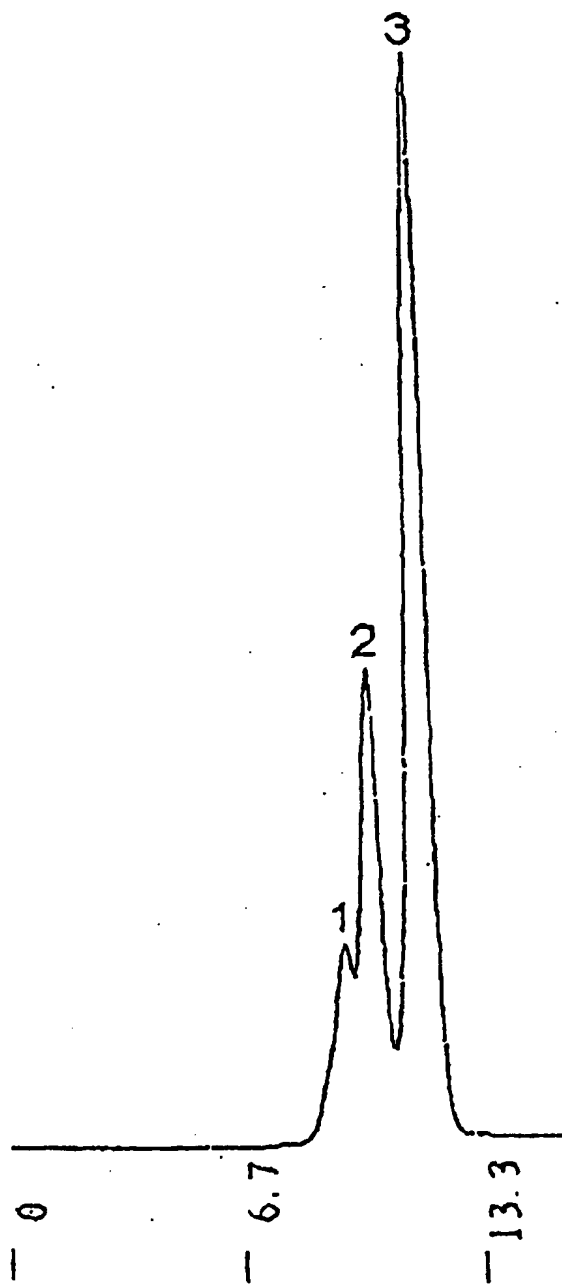


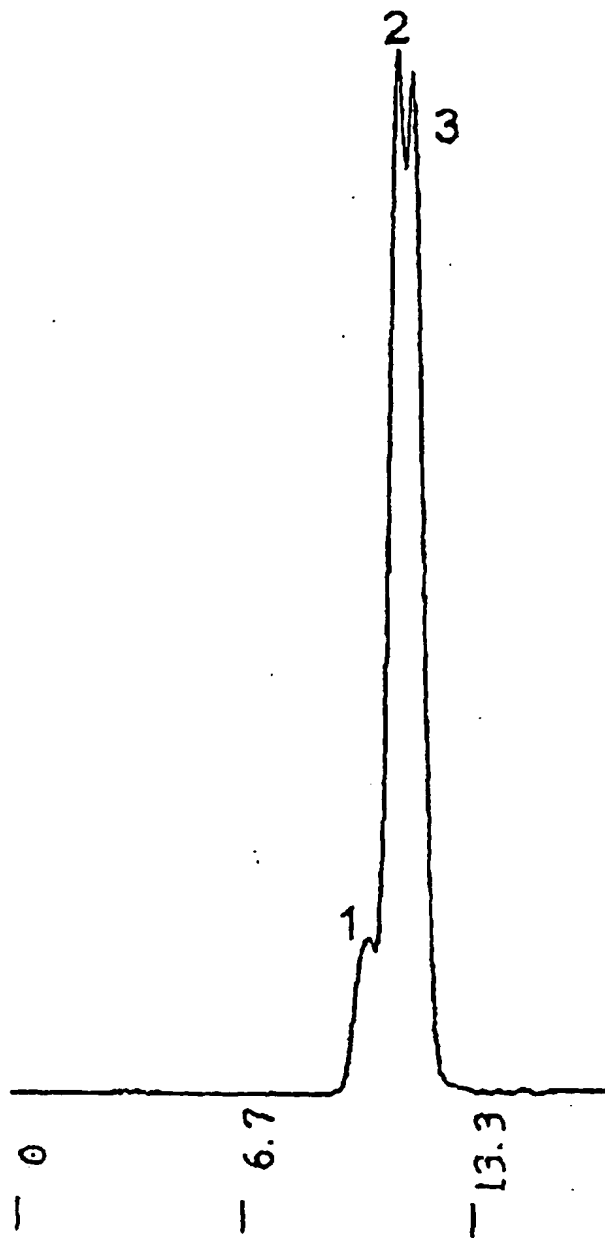


Figure 10



- 1:  $\alpha$ -Galactotetraose or higher oligosaccharides
- 2:  $\alpha$ -Galactotriose
- 3:  $\alpha$ -Galactobiose

Figure 11



- 1: Oligosacchrides containing  $\alpha$ -galactosyl  
of trisaccharide or higher
- 2:  $\alpha$ -Galactosyl glucose
- 3:  $\alpha$ -Galactobiose

Figure 12

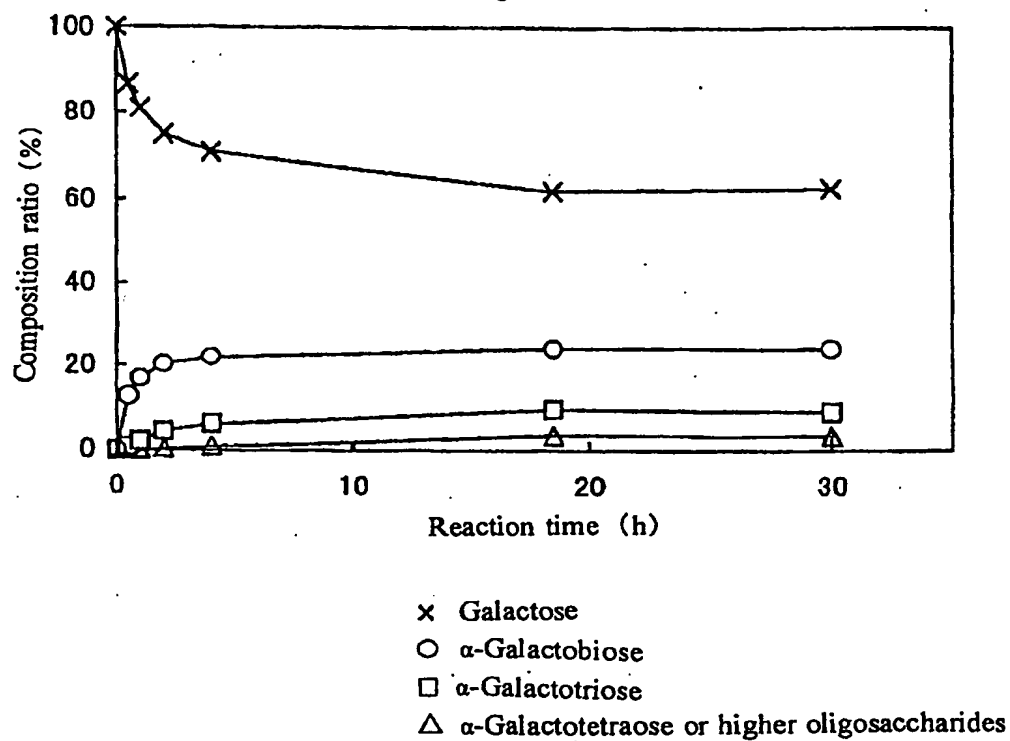
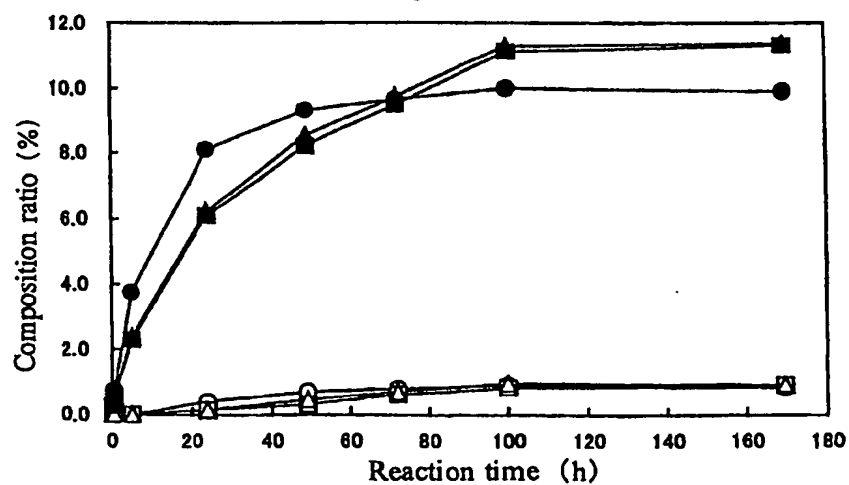
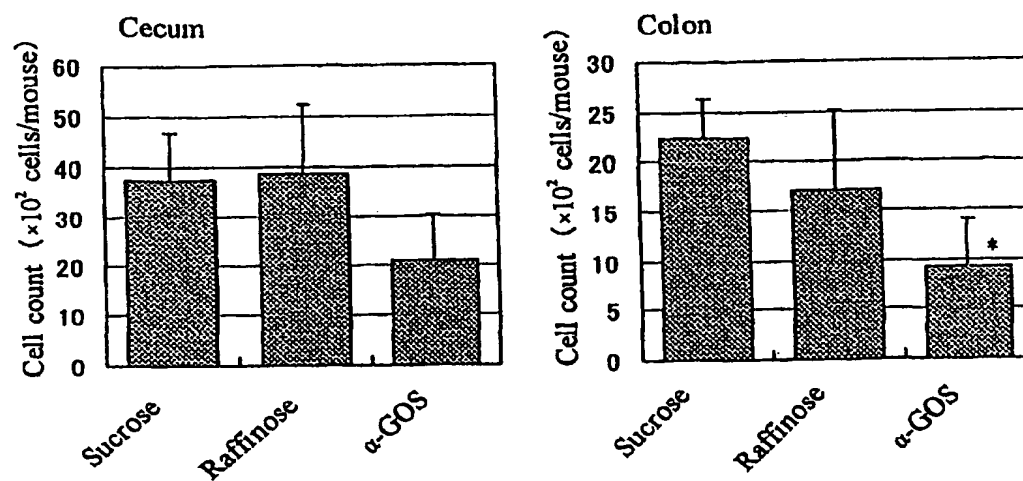


Figure 13



Enzyme in EXAMPLE 1 (● : α-Galactobiose, ○ : α-Galactotriose)  
 Protease B "AMANO" (■ : α-Galactobiose, □ : α-Galactotriose)  
 Nuclease "AMANO" (▲ : α-Galactobiose, Δ : α-Galactotriose)

Figure 14



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/06848

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl. <sup>7</sup> C12P19/14, C12N9/40, C12N1/14  According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl. <sup>7</sup> C12P19/14, C12N9/40, A61K31/33-33/44, C07H1/00-23/00  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS/MEDLINE/WPI (STN) JICST (JOIS)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	JP 5-140178 A (Honen Corp.), 08 June, 1993 (08.06.93) (Family: none)	6, 11-13 1-5, 7 8-10
X Y A	WO 94/23022 A1 (NOVO-NORDISK AS), 13 October, 1994 (13.10.94), & JP 8-508644 A & EP 692024 A1	8-10 1-5, 7 6, 11-13
Y A	JP 5-23183 A (Nihon Shokuhin Kako Ltd.), 02 February, 1993 (02.02.93) (Family: none)	1 2-13
Y A	JP 4-126087 A (Nippon Beet Sugar Mfg. Co., Ltd.), 27 April, 1992 (27.04.92) (Family: none)	1 2-13
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 06 November, 2001 (06.11.01)		Date of mailing of the international search report 27 November, 2001 (27.11.01)
Name and mailing address of the ISA/ Japanese Patent Office  Facsimile No.		Authorized officer  Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/06848

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	MARGOLLES-CLARK E et al., Three alpha-galactosidase genes of <i>Trichoderma reesei</i> cloned by expression in yeast. <i>Eur J Biochem.</i> , 1996, 15 August, 240 (1), pages 104-11.	1 2-13
Y A	EP 192401 A1 (NOVO-NORDISK AS), 27 August, 1986 (27.08.86), & JP 61-187787 A	1 2-13
A	JP 11-240837 A (Nippon Beet Sugar Mfg. Co., Ltd.), 07 September, 1999 (07.09.99) (Family: none)	1-13
X Y A	Tomikatsu HIKARI, "Taikyuu-sei $\alpha$ -Galacto Cytase wo mochiita $\alpha$ -Galacto Oligo-tou no Gousei", Zaidan Houjin Iijima Kinen Shokuhin Kagaku Shinkou Zaidan Heisei 6 nendo Nenpou, (1994), pages 212-216	6,11-13 1-5,7 8-10

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/06848

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

The inventions as set forth in claims 1 to 5, 7 to 10 and 12 pertain to a process for efficiently producing oligosaccharides containing  $\alpha$ -galactosyl, compared with the conventional methods, by using an  $\alpha$ -galactosidase with a specific origin, a specific enzyme to be used in this process and anti-candida compositions produced by the process with the use of this specific enzyme. On the other hand, the invention as set forth in claim 6 pertains to a method involving concentration under reduced pressure in a production process with the use of a general  $\alpha$ -galactosidase. The inventions as set forth in claims 11 and 13 pertain to anti-candida compositions obtained by using a general  $\alpha$ -galactosidase. These inventions can be regarded neither as a single invention nor as a group of inventions so linked as to form a single general inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)